EFFECT OF DIETARY GLYCOMACROPEPTIDE AND CHOLESTEROL ON CORTICAL GANGLIOSIDE- AND GLYCOPROTEIN-BOUND N-ACETYLNEURAMINIC ACID IN YOUNG RATS

by

Susan A. Kary
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Susan E. Carlson, PhD

_____
Committee Chair

Rama Garimella, MSc, MS, PhD

_____
Committee Member

Linda Griffith, PhD, RD, CNSD

_____
Committee Member

_____
Date Defended

The Thesis Committee for Susan A. Kary certifies
this is the approved version of the following thesis:

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_____
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Linda Griffith, PhD, RD, CNSD

_____
Committee Member

_____
Date Approved

Abstract

Background: Sialic acid and cholesterol are present in human milk and accumulate rapidly in the brain during development. Infant formulas contain little sialic acid or cholesterol. Sialic acid and cholesterol supplementation increase cortical ganglioside and glycoprotein sialic acid and cholesterol in animal models, respectively. Dose-response studies and those varying both sialic acid and cholesterol intake are lacking.

Objective: The purpose of this study was to determine the effects of bovine casein glycomacropeptide (CGMP) and dietary cholesterol on cortical ganglioside- and glycoprotein-bound sialic acid accumulation in young rats.

Design/Methods: Dams were fed a nutritionally complete rat diet with 0 or 0.5% cholesterol by weight throughout pregnancy and lactation. Litters were culled to 8 pups on postnatal day (P) 1. Coagulated milk was removed from the stomach of P1 pups and the cholesterol concentration determined. After weaning, two pups from each litter were allocated to one of four diets varying in CGMP (provided approximately 0, 20, 40 and 80 mg sialic acid/kg•d^{-1}) and the same cholesterol concentration as their dams. In the first experiment, the dams' litters were weaned on P17 to the sialic acid-supplemented diets and sacrificed on P32. In the second experiment, litters were weaned on P21 to the diet of their dams. Pups consumed the dams' diets until P24 or P33 and their assigned sialic acid-supplemented diets thereafter. The pups were sacrificed between P38 and P47, 14 days after the initiation of the sialic acid-supplemented diets. On the day of sacrifice, pups were decapitated and their brains immediately frozen on dry ice. Gangliosides and glycoproteins were

extracted from the cortex and the sialic acid concentration determined.

Results: Maternal cholesterol intake increased total milk cholesterol ($p = 0.0009$). In pups sacrificed on P32, CGMP increased cortical ganglioside sialic acid in a dose-response manner (p for trend = 0.007). In the same experiment, dietary cholesterol independently increased ganglioside sialic acid ($p = 0.02$). In pups sacrificed between P38 and P47, cholesterol intake increased glycoprotein sialic acid ($p = 0.030$).

Conclusions: Increased sialic acid and cholesterol consumption during the first 4 weeks of postnatal brain growth influenced brain composition in rats.

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Chapter 1 Introduction

The sialic acid, N-acetylneuraminic acid (Neu5Ac), and cholesterol are important components of the brain that accumulate rapidly during development. All human milk provides Neu5Ac and cholesterol, but infant formulas contain little to none of these biomolecules (1, 2). Among exclusively breast-fed infants, Neu5Ac intake accounts for 0.1% (wt/vol) of the diet in the first two weeks of life (1). Although the concentration of Neu5Ac in human milk exponentially decreases within the first 2 months of lactation, it continues to serve as a significant component of the diet throughout the first 7 months (1). In contrast, infant formulas contain less than 25% of the sialic acid found in mature human milk and include a higher proportion of total sialic acid bound to glycoproteins compared to free oligosaccharides (2). Similarly, human milk contains 10-11 mg of cholesterol per deciliter, while cows' milk-based and soy-based formulas contain 3-5 mg/dL and 0 mg/dL, respectively (3).

Neu5Ac and cholesterol serve several important functions in the brain. Neu5Ac is a structural and functional component of gangliosides and glycoproteins and is involved in a variety of membrane-related events, including cellular recognition, adhesion, and signal transduction (4). A few studies suggest an endogenous source of Neu5Ac is important for proper neural development during infancy, and studies employing the use of animal models demonstrate a positive correlation between the concentration of Neu5Ac in the brain and learning performance (5, 6). In 1980, Morgan and Winick found that intraperitoneal administration of Neu5Ac during the first 30 days of life significantly increased

Neu5Ac incorporation in brain gangliosides and glycoproteins of well-fed and undernourished rat pups and permanently reduced the expected behavioral abnormalities secondary to malnutrition (6, 7). Similar effects have been observed with oral administration of sialic acids (5) and early environmental stimulation (6, 8).

Cholesterol plays an important role in neuroarchitecture and the transmission of neural impulses. It is a key component of neuronal cell membranes, nerve growth cones, and myelin (9). Cholesterol is not uniformly distributed in neuronal cell membranes, but is concentrated in microdomains, called lipid rafts. These heterogeneous areas in the membrane are enriched in gangliosides and thought to function as a platform for signaling pathways (10, 11). Observational studies in humans have linked total serum cholesterol with measures of cognitive function, such as verbal fluency, concentration, and abstract reasoning (12).

Rapid growth in infancy can increase the requirement for exogenous sources of nutrients. It is important to determine if dietary Neu5Ac and cholesterol consumed from milk can influence the accumulation of these compounds in the brain during this critical period of brain growth and development. Previous studies demonstrate that dietary Neu5Ac can increase cortical ganglioside and glycoprotein-bound Neu5Ac (1, 5), but few have investigated the dose-response effects of Neu5Ac supplementation. Studies varying both sialic acid and cholesterol intake are also lacking.

The purpose of this project was to determine if glycomacropeptide, a concentrated source of sialic acid found in bovine milk, and dietary cholesterol influence cortical ganglioside- and glycoprotein-bound Neu5Ac accumulation in

young rats. Three specific research questions were addressed:

1. Does postnatal administration of glycomacropeptide increase the concentration of cortical ganglioside- and glycoprotein-bound sialic acid in developing rats?
2. Does exposure to dietary cholesterol during gestation, lactation and postweaning influence the accumulation of cortical sialic acid?
3. Do quantitative differences in cortical ganglioside- and glycoprotein-bound sialic acid exist among rats administered glycomacropeptide at various maturational stages?

Chapter 2 Review of Literature

Sialic Acids

Sialic acids comprise a large family of nine-carbon α-keto acids derived from 3-deoxy-non-2-ulosonic acid (13). The structure of sialic acids assumes a chair conformation and includes a deoxy moiety adjacent to the anomeric carbon, a glycerol branch at C6, and a carboxylate group at C1 (13). Under physiological conditions, the carboxylate group confers a negative charge on the molecule and characterizes it as a strong organic acid (pK_a 2.2) (14, 15).

Naturally occurring sialic acids can be divided into three major types, based on the functional group located at C5. The substituent can be an acetamido, a glycolyl, or a hydroxyl group, forming N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxy-D-glycero-D-galacto-nonoic acid (KDN), respectively (15, 16) **(Figure 1)**. The hydroxyl groups located on carbons 4, 7, 8, and 9 can be further modified by phosphorylation, methylation, acetylation, lactylation or sulfonation, producing over 50 structurally distinct compounds with unique properties (13, 15, 16). Unsaturated sialic acids and internal anhydro linkages have also been observed (15).

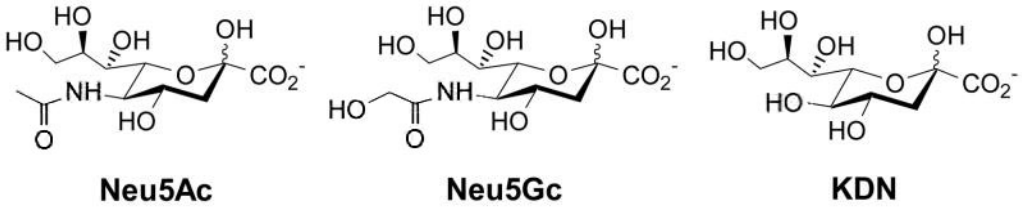

Figure 1. Three basic forms of naturally occurring sialic acids.

Sialic acids are commonly located on the distal end of glycan chains conjugated with lipid or protein scaffolds. The penultimate glycan residue typically consists of glucose, galactose, N-acetyl-galactosamine, or N-acetyl-glucosamine, linked to a sialic acid through α-(2-6) or α-(2-3)-linkages (13). Although sialic acids are primarily present as monosialyl residues, disialic residues may be formed at the distal end of glycan chains through α-(2-4), α-(2-8) or α-(2-9)-linkages (13). Linear homopolymeric structures of sialic acid attached to glycoproteins have also been observed in several species (13, 15, 16), with chain lengths varying from 8 to 200 residues. These negatively charged, voluminous polymers vary in both monomer type and linkage (16). Thus far, α-(2-8), α-(2-9), alternating α-(2-8)/α-(2-9), and α-(2-5)$O_{glycolyl}$-linkages have been described (16).

Due to their outmost positioning and hydrophilic character, sialic acids exert a physiochemical force on their environment and mediate several important cellular recognition and adhesion processes (14, 17). Sialic acid residues bind and transport positively charged molecules, attract and repel cells, serve as antigenic determinants, and function as an antiproteolytic shield for glycoproteins (14, 15). They are necessary components of receptors for endogenous substances, such as hormones and cytokines, as well as pathogenic agents, including toxins, viruses, bacteria, and protozoa (14, 15). Sialic acids function as ligands for receptors, such as lectins, selectins, and members of the sialoadhesion family, mediating a variety of processes involved in the immune response and cell migration (13, 14, 15). They also participate in the carbohydrate-protein interactions responsible for promoting cell

growth, cell differentiation, fertilization, and oncogenesis (13).

N-acetylneuraminic Acid

N-acetylneuraminic acid (Neu5Ac) is the most abundant sialic acid in nature (4, 14, 17) and is found in high concentration in neural cell membranes and human milk (1, 2). Its synthesis is compartmentalized within the cell and begins in the cytosol (14) with the formation of N-acetylmannosamine (ManNAc) from UDP-N-acetylglucosamine (UDP-GlcNAc) (17) **(Figure 2)**. This reaction, catalyzed by

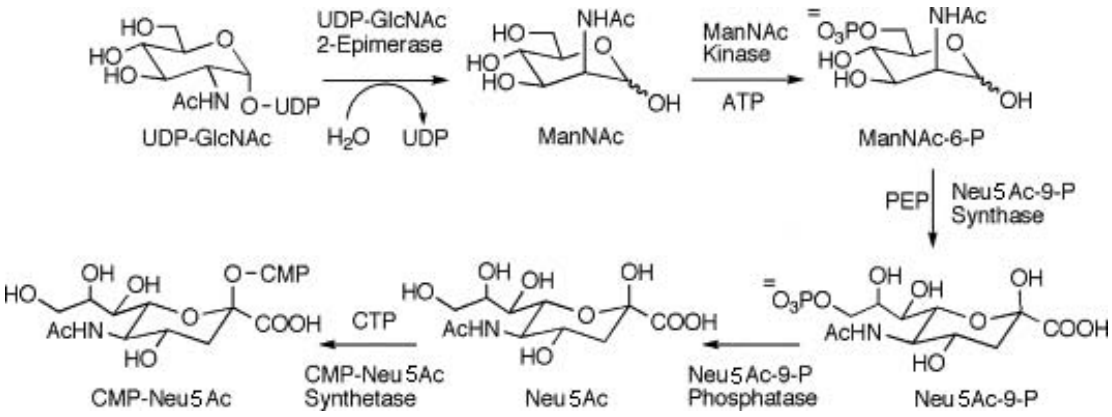

Figure 2. The biosynthesis of CMP-Neu5Ac in mammals.

UDP-GlcNAc 2-epimerase (GNE), both hydrolyzes the glycosidic phosphate bond and inverts the stereochemistry at C2 (17). In mammals, ManNAc is phosphorylated by ManNAc kinase to produce ManNAc 6-phosphate (ManNAc-6P), and Neu5Ac-9-P synthase catalyzes the condensation of ManNAc-6P with phosphoenolpyruvate to

give Neu5Ac 9-phosphate (Neu5Ac-9P) (17). It is worth noting that the human form of Neu5Ac-9-P synthase can also accept mannose 6-phosphate as an alternative substrate in this pathway, generating KDN 6-phosphate (17). This is not true for all eukaryotic forms of the enzyme, including that of the rat (17).

After dephosphorylation of Neu5Ac-9P by Neu5Ac-9-P phoshatase, Neu5Ac is transported to the nucleus, where it is converted to its active form (14, 17). This is accomplished through the addition of a cytidine monophosphate (CMP) residue from cytidine triphosphate (CTP) by CMP-Neu5Ac synthetase (14, 17). CMP-Neu5Ac is then translocated into the Golgi apparatus or the endoplasmic reticulum, where it serves as the substrate for all sialytransferases. These enzymes transfer Neu5Ac to the oligosaccharide chain of nascent glycoproteins and glycolipids (14, 17). Interestingly, CMP-Neu5Ac is the only sialic-acid containing molecule where the anomeric configuration is β (13, 14).

Two other metabolic pathways for the synthesis of Neu5Ac are known, including the condensation of enolpyruvate phosphate and ManNAc, catalyzed by Neu5Ac synthase, and an aldol reaction between pyruvate and ManNAc, catalyzed by acylneuraminate pyruvate-lyase (18). The enzyme that facilitates the first reaction pathway has only been described in pathogenic strains of bacteria that display sialic acids on their cell surface to mimic mammalian cells and conceal antigenic sites, including *Neisseria meningitidis*, *Escherichia coli* K1, and *Campylobacter jejuni* (17, 18). The latter enzyme, acylneuraminate pyruvate-lyase, is present in both bacteria and mammals. However, bacteria that express acylneuraminate pyruvate-lyase do not

synthesize Neu5Ac, and this enzyme is absent from mammalian tissues that continuously produce sialic acids (18). It is thought that acylneuraminate pyruvate-lyase is involved in catabolic, rather than anabolic reactions (18). This idea is supported by the position of the equilibrium of the reaction, which is unfavorable for Neu5Ac synthesis (18).

The primary enzymes involved in Neu5Ac catabolism are carbohydrate-specific sialidases (14). Sialidases hydrolyze the glycosidic linkage between Neu5Ac residues and the penultimate sugar of the oligosaccharide chain of glycoproteins and glycolipids (14). In higher animals, glycoconjugates are generally taken up by receptor-mediated endocytosis. After fusion of the endosome with a lysosome, the terminal Neu5Ac residues are removed by lysosomal sialidases (14). Free Neu5Ac molecules are transported through the lysosomal membrane into the cytosol (14). Here, they can be further degraded by acylneuraminate lyases or recycled by activation and transferred onto another nascent glycoprotein or glycolipid molecule (14). Alternatively, Neu5Ac residues can be removed from glycoproteins and glycolipids on the cell surface by membrane-bound sialidases (14).

Ganglioside-bound N-acetylneuraminic Acid

As previously mentioned, sialytransferases located in the Golgi apparatus and endoplasmic reticulum transfer Neu5Ac residues to the oligosaccharide chain of nascent glycoproteins and glycolipids. Neu5Ac serves as a structural and functional component of both glycoproteins and glyolipids.

Glycolipids contain one or more monosaccharide residues covalently bound to a hydrophobic moiety, such as an acylglycerol, a sphingoid, a ceramide, or a prenyl phosphate, by a glycosidic linkage (19, 20). Glycolipids are membrane-anchored and the carbohydrate structures are exposed at the cell surface (21). To date, more than 300 glycolipids have been isolated and characterized according to their lipid and carbohydrate moieties (19, 20). Those containing one or more sialic acid residues belong to a family of acidic glycosphingolipids and are designated sialoglycosphingolipids or gangliosides (19).

Gangliosides are commonly named according to a convention established by Lars Svennerholm (1963), in which a ganglioside is specified by the letter G followed by M, D, T, or Q, indicating the presence of one, two, three, or four sialic acid residues, respectively (19). A number, which initially referred to the migration order of the ganglioside, is then assigned to each individual compound (19). These designations are not systematic and do not correlate with the molecular structure of the ganglioside (19). For example, the structure of GD3 is Neu5Acα8Neu5Acα3Galβ4GlcCer. Nevertheless, Svennerholm's abbreviations are used because they are short and well understood (19).

De novo synthesis of gangliosides begins with the formation of ceramide at the cytosolic leaflet of the endoplasmic reticulum membrane (22, 23, 24). Serine palmitoyl transferase catalyzes the condensation of L-serine and a fatty acyl coenzyme A, typically palmitoyl coenzyme A, to form 3-ketosphinganine (22, 23). This molecule is reduced to D-erythro-sphinganine by 3-ketosphinganine reductase

and acylated to dihydroceramide by an N-acyltransferase (22, 23). Ceramide is produced through the desaturation of dihydroceramide in a reaction catalyzed by dihydroceramide desaturase (22, 23).

Ceramide is a lipid biomodulator of various cellular functions and serves as the membrane anchor of all glycosphingolipids and sphingomyelin (22, 23, 24). To continue ganglioside biosynthesis, ceramide is transferred to the cytosolic leaflet of the Golgi apparatus with the aid of the ceramide transfer protein (CERT) (23). Here, a glucose residue is β-glycosidically linked to the 1-position of ceramide, forming glucosylceramide (GlcCer) (21, 22, 23). This reaction is catalyzed by glucosylceramide synthase and utilizes uridine diphosphoglucose as the glycosyl donor (21, 22, 23). Alternatively, ceramide may be galactosylated by galactosyltransferase to form galactosylceramide. This precursor is needed to synthesize sulfatides and GM4, a major ganglioside of the myelin membrane (22, 23). At this point, GlcCer can reach the plasma membrane by direct transport or be further modified by glycosylation on the luminal leaflet of the Golgi membranes (22). The latter is necessary for ganglioside synthesis. Galactosyltransferase-I catalyzes the addition of a galactose moiety from uridine diphosphogalactose to glucosylceramide, producing lactosylceramide (LacCer) (21, 22, 23).

With the exception of GM4, all gangliosides are synthesized through the stepwise addition of monosaccharide residues onto LacCer from sugar nucleotide donors imported from the cytoplasm (22, 23, 25). Addition of the first Neu5Ac residue converts LacCer to GM3 in a reaction catalyzed by either GM3-synthase or

sialyltransferase I (ST-I) (22, 23). GM3 is the precursor of most complex brain gangliosides, and subsequent addition of Neu5Ac residues generates gangliosides GD3 and GT3 (23). These reactions are catalyzed by structurally and genetically distinct enzymes, termed GD3-synthase or ST-II and GT3-synthase or ST-III, respectively (23). Collectively, GM3, GD3, and GT3 are known as the hematosides or sialyl-lactosylceramides. Together with LacCer, they serve as the entry substrates for the synthesis of complex gangliosides in the a-, b-, c-, and 0-series pathways (22, 23). To date, about 200 gangliosides differing in carbohydrate constituents have been identified, but those from the 0- and c-series are only found in trace amounts in adult human tissues (22, 23).

Gangliosides are typical components of vertebrate cells and are asymmetrically located in the outer molecular leaflet of the plasma membrane (26, 27, 28). Although they are present in virtually every cell type, they are especially concentrated in the central and peripheral nervous systems (CNS and PNS) (23, 26, 27). Gangliosides are most abundant in areas predominating in neuronal cell bodies, such as the cerebral gray matter, cerebellar gray matter, and caudate nucleus (29). The lowest concentrations occur in areas consisting largely of myelinated fiber tracts and glial cells, as in the cerebral white matter, pons, medulla, and corpus callosum (29). This concentration differential is observed due to the specific localization of gangliosides in dendritic and axonal neuronal membranes and their synaptic connections (26, 28, 29). Although some gangliosides are present in the myelin membrane, they are virtually absent from glial cells, producing a 10-fold difference in

ganglioside-bound Neu5Ac between gray and white matter (29).

Studies using monoclonal antibodies and thin layer chromatography have revealed that single ganglioside species are specifically distributed in nervous tissues and signaling pathways (30, 31). For example, the gray matter of the mammalian brain primarily consists of GM1, GD1a, GD1b, GT1b, and GQ1b, whereas the white matter predominantly contains GM1 and GD1a (31). Similarly, GM1 accounts for 15% of the total ganglioside in motor nerve myelin, while only trace amounts are observed in sensory nerve myelin (31). In 1999, Vorwerk et al. discovered that the ceramide composition of GD1a, GD1b, and GM1 also varies between human motor and sensory nerves and, later, published data supporting a link between the ganglioside distribution patterns in the human spinal cord and function (31).

Gangliosides are known to play crucial modulatory roles in several membrane-related phenomena, including cellular recognition, adhesion, interaction, and signal transduction (23, 26, 30). Although some of the mechanisms by which gangliosides interact with their surrounding environment are not well understood, the concept of a multifunctional role for gangliosides seems suitable to explain their different functional implications (23, 26).

The Neu5Ac residues, and the other oligosaccharide components of gangliosides that protrude from the cell surface, possess the potential to interact with external ligands (26). This allows gangliosides to function as receptors for bioactive molecules and mediators of cellular recognition and adhesion (32). At least 11 Neu5Ac-binding proteins, or lectins, have been identified in humans (33, 34).

Together, they are designated as members of the Siglec family (33, 34). While some are highly specific for a single type of Neu5Ac linkage, others appear less selective (33). A common example is the binding of myelin-associated glycoprotein (MAG) with gangliosides GD1a and GT1b. MAG is a quantitatively minor protein found on the innermost periaxonal myelin membrane and functions as a myelin-stabilizing factor and inhibitor of nerve regeneration (33, 35). In order to elicit its physiological effects, MAG must bind to the Neu5Ac residues of GD1a and GT1b on the neuronal membrane (33, 35). Gangliosides exhibiting other Neu5Ac linkages fail to support MAG-mediated adhesion (33, 35). Studies have demonstrated that mice lacking the ability to synthesize GD1a and GT1b display progressive neurodegenerative abnormalities, including axon degeneration and demyelination in the CNS and PNS (33, 35). Thus, this ganglioside-specific adhesion process is required for long-term axon stability (33, 35). Recent evidence suggests that GT1b can also facilitate the interaction between MAG and the Nogo-66 receptor 1, a neuronal protein required for acute growth cone collapsing (36, 37), providing another example of ganglioside-mediated cellular communication. Other important Neu5Ac-binding lectins include the B-lymphocyte surface receptor CD22, CD33, and sialoadhesin (33, 34).

Gangliosides also exert a physiochemical force on extracellular substances (26). A noteworthy example is the interaction between Neu5Ac residues on astrocytic gangliosides and extracellular matrix-associated laminin. Laminin is a large extracellular glycoprotein that has been implicated in the morphogenesis of the nervous system (38). In adult basement membranes, laminin is spatially separated

from the bulk extracellular matrix and is organized in a continuous mesh-like network (38). During embryonic development, however, laminin appears as cell-surface-associated deposits or is arranged in large polymeric aggregates scattered within the extracellular space (38). These morphologies tend to restrict or permit neurite outgrowth, respectively (38). Observations from in vitro and cell-free studies suggest that gangliosides on the surface of astrocytes guide the formation of these laminin polymers (38). Removal of Neu5Ac residues on embryonic monolayers by neuraminidase treatment leads to the immediate release of matrix-associated laminin and converts the laminin matrix to a more restrictive form after reassembly (38). A related phenomenon is observed in artificial lipid films. Films containing GT1b or a mixture of gangliosides favor a more permissive morphology, while those consisting of phophatidylcholine or GM1 tend to inhibit the growth of neurites (38). This suggests that the number of Neu5Ac residues present on the surface of astrocytes plays a prominent role in directing the assembly of laminin matrices (38).

In the plasma membrane, gangliosides are believed to segregate into microdomains enriched in glycosylphosphatidylinositol (GPI)-anchored proteins, sphingomyelin, and cholesterol (23, 26, 39, 40). These domains are variously referred to as lipid rafts, detergent-resistant membranes, and detergent-insoluble glycosphingolipid-enriched domains (40). Although the exact function of lipid rafts is not fully understood, they appear to change the biophysical properties of the lipid bilayer and facilitate ganglioside interaction with membrane proteins, such as receptors, enzymes, ion channels, and ion pumps (23, 26, 39). For example,

ganglioside GD1a has been shown to increase the excitability of voltage-dependent batrachotoxin-modified sodium channels derived from rat brain (41). This equips neurons with an additional tool for regulating their excitability and is thought to ensure proper synaptic transmission (41). Gangliosides also modulate calcium exchange between the extracellular space and synaptoplasm, which may contribute to memory formation through stabilization of synaptic contacts (41, 42).

By interacting with functional proteins, gangliosides may also facilitate metabolic second messenger production, signal transduction, and cellular responses based on protein phosphorylation and dephosphorylation processes (23, 26, 32, 43). Gangliosides have the capacity to produce metabolic second messengers of sphingoid nature (26). Ceramide and sphingosine are potent regulators of protein kinase C and other protein kinases and enzymes involved in signal transduction (26). A possible mechanism for the formation of these second messengers is the intra-lysosomal breakdown of membrane-bound gangliosides after ligand-mediated endocytosis (26).

Glycoprotein-bound N-acetylneuraminic Acid

Sialytransferases in the Golgi apparatus and endoplasmic reticulum also transfer Neu5Ac residues to the oligosaccharide chain of nascent glycoproteins. Glycoproteins are macromolecules which consist of heteropolymerized carbohydrate sub-units covalently attached to amino acids of a polypeptide backbone (44, 45). Glycoproteins are ubiquitous in all forms of life, and protein glycosylation is a typical post-translational modification of most enzymes, receptors, antibodies, hormones,

cytokines, and structural proteins (44). The carbohydrate, or glycan, can range in size from one to hundreds of monosaccharides and may form linear or branched polymers (44). The number of glycans present can also vary greatly between proteins (44).

A striking feature of almost all glycoproteins is the polymorphism associated with their glycan moieties (44). Individual molecules of a given glycoprotein can carry different oligosaccharides at the same glycosylation site of the protein backbone (44). This phenomenon is known as microheterogeneity, and the resulting variants are referred to as glycoforms (44). Microheterogeneity was originally thought to be the result of a lack of fidelity in glycoprotein synthesis (44). Recent evidence suggests that under constant physiological conditions, the set of observed glycoforms is reproducible and highly regulated (44). The population of glycoforms has also been shown to change during cell growth, cell differentiation, and malignant transformation, suggesting that defined sets of glycoforms are required for normal functioning (44).

Three major types of linkages covalently attach glycans to the amino acids of a polypeptide backbone (44). These include N-glycosidic linkages, O-glycosidic linkages, and GPI anchors (44). N-glycosidic linkages result from the transfer of a common core pentasaccharide from the donor molecule dolichol pyrophosphate to the amide nitrogen atom in the side chain of asparagine (21, 44, 46). After trimming of the core pentasaccharide, nucleotide-activated monosaccharides are sequentially added to the terminal mannose residues by glycosyltransferases located in the endoplasmic reticulum and Golgi apparatus (21, 45, 46). Glycosyltransferases

display distinctive substrate-, cell-type-, and developmental-stage-specificity, producing glycans with great diversity (21, 45).

The core pentasaccharide of N-glycosidic linkages, which consists of three mannose and two N-acetylglucosamine residues, can only be transferred to an Asn-X-Ser or Asn-X-Thr sequence, where X can be any residue, except proline (21, 44, 46). This allows potential glycosylation sites to be detected within the amino acid sequence (46). However, not all potential sites are glycosylated, and those selected depend on both the structure of the protein and the cell type in which it is expressed (44, 46). For example, mechanistic studies demonstrate that the enzyme responsible for forming N-glycosidic linkages, oligosaccharyl transferase, may only glycosylate peptides which are able to adopt the Asx-turn conformation (44).

O-glycosidic linkages typically occur between the side chain hydroxyl group of serine or threonine and the reducing end of monosaccharide residues (21, 44, 46). Although less common, the side chain hydroxyl groups of hydroxyproline, hydroxylysine, and tyrosine can also support O-glycosidic linkages (44). Unlike N-glycosylation, biosynthesis of O-glycan chains takes place exclusively in the Golgi apparatus and begins with the addition of single nucleotide-activated monosaccharides to the protein backbone, producing a variety of carbohydrate-protein linkages (21, 44, 46).

GPI anchors are structurally unrelated to N-glycosidic and O-glycosidic linkages and involve the attachment of a glycolipid to a nascent protein during post-translational modification. Specifically, GPI anchors link the C-terminal amino acid

of a protein to an oligosaccharide attached to phosphatidylinositol via ethanolamine phosphate (44). This reaction is catalyzed by GPI transamidase in the endoplasmic reticulum and occurs rapidly upon completion of protein translation and translocation (47). GPI anchors are important because they provide a means for anchoring proteins with a wide variety of structures and biological functions to the extracellular surface of the plasma membrane (44, 47).

The most common monosaccharides incorporated into glycoproteins include galactose, glucose, mannose, N-acetylegalactosamine, N-acetylglucosamine, L-fucose, and Neu5Ac (44, 45). Neu5Ac residues are invariably located at the terminal end of glycan chains, with galactose or N-acetlygalactosamine serving as the penultimate monosaccharide (45). This outermost positioning is observed because Neu5Ac residues are rarely attached to the polypeptide backbone (39), and no further chain elongation is possible after the addition of Neu5Ac, except for the formation of Neu5Ac polymers (44). In the brain, N-glycans and O-glycans predominantly carry α-(2,3)-linked Neu5Ac, with little or no α-(2,6)-linked Neu5Ac, while Neu5Ac polymers typically consist of α-(2,8)-linkages (21). Because Neu5Ac is a common component of many glycoproteins and microheterogeneity is abundant, only a summary of the general location and functions of Neu5Ac-containing glycoproteins, or sialoglycoproteins, will be presented.

Many glycoproteins are located in the cytoplasm, subcellular organelles, and extracellular fluid, while others, including sialoglycoproteins, are components of the plasma membrane (44, 45, 46). Hydrolysis experiments have revealed that

sialoglycoproteins and gangliosides share a similar distribution pattern in the CNS (29). Like gangliosides, sialoglycoproteins are most abundant in areas predominating in neuronal cell bodies, including the cerebral gray matter, cerebellar gray matter, and caudate nucleus, while the lowest concentrations occur in areas consisting primarily of myelinated fibre tracts and glial cells, as in the cerebral white matter, pons, medulla, and corpus callosum (29). Relative to sialoglycoproteins, gangliosides are slightly more concentrated in the gray matter (29). This observation may be due to the activity of membrane-bound sialidase (14, 29). While the regional distribution of sialidase parallels the distribution of total Neu5Ac, its activity is much greater in the gray matter (29). Roukema and Heijlman (1970) suggest gangliosides contain less hydrolyzable Neu5Ac or are less accessible to sialidase than sialoglycoproteins, due to the aggregation of gangliosides on the plasma membrane (29). This would result in the preferential hydrolysis of the Neu5Ac residues attached to sialoglycoproteins, producing a lower ratio of sialoglycoproteins to gangliosides in the gray matter.

While the regional distribution of sialoglycoproteins is similar to that of gangliosides, they do not appear to share the same subcellular localization (29, 48). Gangliosides and sialoglycoproteins primarily occur in the crude mitochondrial and microsomal fractions sedimented from rat whole brain (29, 48). Exposure of the crude mitochondrial fraction to osmotic shock before density-gradient centrifugation reveals that the largest percentage of gangliosides and sialoglycoproteins is found in synaptosomes (29, 48). If the synaptosome-enriched fraction is subjected to a more extensive fractionation, sialoglycoproteins sediment into more dense subfractions,

while gangliosides appear more evenly distributed (29, 48). This differential sedimentation suggests that sialoglycoproteins and gangliosides are found in different subcellular locations (29, 48).

There is no single unifying function for the carbohydrates present in glycoproteins (44, 49). While some participate in physiological and pathological recognition events, others modify the chemical, physical, and biological properties of the proteins to which they are attached (44, 49). The same glycan may also display distinct functions at separate locations on a given protein, or in different cell types or developmental stages (49). In a manner similar to the single oligosaccharide unit of gangliosides, the glycan moieties of membrane-bound sialoglycoproteins, such as prions, integrins, CD34, and the neural cell adhesion molecule, penetrate through the lipid bilayer and extend into the extracellular fluid (39, 45). Here, Neu5Ac residues regulate several cellular adhesion processes, acting as a lubricant that modulates the interaction between sterically masked ligands (45, 50). For example, the α-(2,6)-linked, Neu5Ac residues of integrins serve as antiadhesion molecules and mediate the migration and differentiation of neural progenitors derived from embryonic striatum (39). Investigators suggest these biological functions are conferred by the anionic character of Neu5Ac, which may hinder adhesion by electrostatically repelling other ionized residues on nearby cells (45, 50). Alternatively, the negative charge may function as a barrier to cellular interaction, blocking access to adhesion sites located beneath the Neu5Ac residues on the cell surface (44, 45). This observation may help account for the ability of Neu5Ac to increase the stability of glycoproteins (50). The

residues may cover functionally important areas of the protein, protecting the peptide backbone from recognition by proteases and antibodies (44, 49).

In addition to the chemical structure of Neu5Ac, the context of its presentation is also critical to its function (39). A single Neu5Ac residue has little biological activity, but achieves its specificity through multivalency and the clustering of the residues attached to a specific sialoglycoprotein (39). An example of cluster-dependent function is provided by CD34, a stem cell antigen expressed by proliferating resident microglia in response to acute neural injury (39, 52). The N terminus of CD34 is heavily glycosylated with Neu5Ac and protrudes a considerable distance from the cell membrane (53). This region is thought to adopt the conformation of an extended rod, forming a cellular halo of Neu5Ac-dependent negative charge (53). Together, the Neu5Ac residues possess an anti-adhesive quality and function as a barrier to cell-cell interaction (53). Ganglioside-bound Neu5Ac also requires clustering to elicit a biological effect and is achieved through the aggregation of gangliosides in lipid rafts (39).

A sialoglycoprotein that plays a particularly important role in brain development and neuronal plasticity is the neural cell adhesion molecule (NCAM). NCAM is a member of a large family of membrane-bound glycoproteins that share structural motifs related to immunoglobulin and fibronectin type II domains (54). It is unique among recognition molecules in that its adhesive and signal transduction functions are modulated by polysialic acid (PSA), a linear homopolymer containing up to 200 α-(2,8)-linked Neu5Ac residues (21, 55, 56). Unlike most carbohydrates

displayed on the cell surface, PSA is exclusively attached to NCAM, an observation supported by the nearly complete absence of PSA in NCAM-deficient mice (56). PSA is added to NCAM post-translationally by one of two Golgi-associated polysialytransferases, namely ST8SiaIV (PST) and ST8SiaII (STX) (21, 56). These enzymes attach PSA to two asparagines in the Ig5 module of the extracellular portion of NCAM (21, 56). While PST is primarily expressed in the adult, STX is more highly expressed during development (21). After developmental events cease, NCAM typically shifts from a PSA-rich to a PSA-poor form (21, 54, 56, 57). Interestingly, STX continues to be expressed and PSA is retained in areas of the adult brain that display the capacity for morphological and physiological plasticity, including the neuroblasts of the rostral migratory stream and the innermost granule cell layer of the hippocampal dentate gyrus (21, 39, 54, 57). Immature neurons in the dentate gyrus mature and integrate into the neuronal network in an activity-dependent manner (21). Once these neurogenic stem cells differentiate, their expression of PSA is reduced (21). Remarkably, the expression of PSA on NCAM in neuroblasts is enhanced after training in spatial memory tasks, when new connections might need to be formed or when a synapse might undergo morphological changes (21). Based on these activities, it is not surprising that NCAM is connected with learning and memory consolidation (39, 56). The loss of PSA eliminates the anti-adhesive character of this surface protein and, subsequently, appears to halt the migration of neuroblasts to the outer part of the granular layer and promote the stabilization of synaptic contacts (21, 54). Similarly, removal of PSA from NCAM by endosialidase

N alters the outgrowth or sprouting of axons, neuronal and glial migration, axonal pathfinding, axon branching, and fasciculation (21, 39, 54).

Developmental Regulation of Gangliosides and Sialoglycoproteins

The expression of ganglioside- and glycoprotein-bound Neu5Ac in the nervous system is developmentally regulated and closely related to the differentiation state of the cell (21, 23). During development, gangliosides undergo characteristic changes in content and composition (58, 59). The total concentration of ganglioside-bound Neu5Ac in the forebrain increases in two distinct phases (59). In humans, the first phase occurs between the 13th and 30th week of gestation, and the second occurs from birth to 9 months of age (59). These phases correspond to the time of rapid multiplication of neurons and the extensive establishment of micro-neuronal interconnections, respectively (59). The concentration of ganglioside-bound Neu5Ac in the cerebellum also increases from birth to 9 months of age (59). In contrast, no appreciable change is observed in the brainstem after 30 weeks of gestation (59). Nine months after birth, the total concentration of ganglioside-bound Neu5Ac in the human brain reaches the adult level and begins to plateau (59).

The composition of gangliosides in the brain also changes during development and is regulated by the activities of glycosyltransferases through gene transcription and posttranslational modification (23, 58, 59). After birth, GM1, GD1a, GD1b, and GT1b account for 90% of the total gangliosides found in the human brain (59). At the 15th week of gestation, GT1b accounts for approximately 38% of the total

gangliosides in the forebrain on a molar basis, while GD1a accounts for about 26% (59). The proportion of GD1a increases rapidly until the 20th week of gestation and more slowly up to the 35th week (59). This is followed by a rapid increase to term, when GD1a accounts for roughly 70% of the total gangliosides (59, 60). After birth, this proportion decreases to about 50% and remains stable through 26 months of age (59). In contrast, the molar percentage of GT1b decreases to 10% at birth and remains constant through the first two years of life (59, 60). The fraction of GD1b falls from 20% at 13 weeks of gestation to 5% at term, and then increases again to the adult level of 15% by 9 months of age (59). After an initial decrease, GM1 increases to a level of 25-30% one year after birth and remains stable thereafter (59).

A similar pattern is observed in the cerebellum, and the contributions of GD1a and GT1b appear to change reciprocally throughout development (59). However, GT1b serves as the predominant fraction in the cerebellum, rather than GD1a (59). Just as no appreciable change in concentration is observed in the brainstem after 30 weeks of gestation, the ganglioside pattern also remains constant (59). These maturational changes appear to be functionally involved in the control of axonal and dendritic outgrowth, synaptogenesis, and the establishment of cell contact (58, 60).

The overall pattern of gangliosides in the brain approaches that of the adult 2 years after birth (59). As adults mature, the ganglioside composition shifts to a more polar pattern, due to the relative increase in GQ1b, GT1b and GD1b and decrease in GD1a and GM1 (58). With the exception of GQ1b, the absolute concentration of all gangliosides decreases with age (58). In a study on 9 neurologically normal males

ranging from 25 to 85 years of age, Segler-Stahl et al. found a decrease in ganglioside-bound Neu5Ac from 1,070 to 380 μg/g of fresh brain tissue (58). This may help account for the progressive degeneration of myelin sheaths, deterioration of dendrites, and loss of neurons and synapses observed in the aging brain (58).

The concentration of sialoglycoproteins appears to increase at the same time as gangliosides and correlates with the outgrowth of cell processes (61, 62). In rats, the most rapid increase in sialoglycoproteins occurs between 4 and 18 days after birth (61, 62). Evidence suggests the rate of sialoglycoprotein biosynthesis during postnatal development is not limited by the activities of CMP-N-acetylneuraminic acid synthetase or glycoprotein sialytransferase, but may depend on the production of endogenous acceptors, or the precursors of sialoglycoproteins (62).

As mentioned in the discussion of NCAM, PSA is highly expressed in the embryo and neonate and exhibits a decreased and more restricted expression in adults (55, 57). This observation is partly due to the expression of the polysialytransferases, PST and STX. Both enzymes are prevalently expressed during development, starting from E8-E9 in rodents, and are downregulated in the adult (56). While STX declines dramatically after birth, PST declines more gradually and is continuously expressed at lower levels through adulthood (56). Similarly, NCAM begins to be expressed at the time of neural tube closure and remains detectable at lower levels in the adult (55).

The expression of PSA-NCAM also fluctuates with circadian rhythms and the ovarian cycle (56). The concentration of PSA in the mammalian suprachiasmatic nucleus, a structure responsible for maintaining circadian rhythms synchronized to

the light/dark cycle, fluctuates rhythmically and is characterized by high levels during the day and low levels at night (56). Studies carried out on the Syrian hamster have revealed that a 90% reduction in PSA occurs during the mid-to-late dark phase, and maximum concentration is reached 1 hour after the lights are turned on (56). During the proestrous phase of the ovarian cycle, PSA-NCAM becomes more heavily associated with axon terminals of the gonadotropin-releasing hormone (GnRH) neurons in the glial processes and median arcuate nucleus (56). These structures are a part of the GnRH neurosecretory system and undergo dynamic structural and functional changes during the ovarian cycle (56).

Interestingly, the expression of PSA-NCAM also fluctuates during lactation. Compared to virgin rats, PSA-NCAM dramatically decreases in the neurohypophysis and supraoptic nuceli during lactation (56). This decrease is not associated with modifications in the amount of NCAM present or changes in the splicing pattern of NCAM mRNAs. It is solely dependent on the expression of PSA, and the concentration of PSA-NCAM returns to its initial value after weaning (56).

N-acetylneuraminic Acid in Infancy

Rapid growth and development of the brain in infancy may place exceptional demand on the endogenous synthesis of Neu5Ac (2, 4, 39). While the livers of all mammals have the capacity to synthesize Neu5Ac from simple sugar precursors, those of newborn infants are relatively immature, and the activity of the rate-limiting enzyme, GNE, is low during the neonatal period (2, 5, 39). This may limit de novo

synthesis, causing Neu5Ac to become a conditionally essential nutrient during this critical period of neurological development (2, 4, 39).

It is well known that human milk serves as an endogenous source of Neu5Ac after birth (1, 2, 5, 63, 64). Evidence also suggests a significant amount of Neu5Ac is provided through the placenta (63). In a study of 219 pregnant women, Alvi et al. found a graded and significant increase in the mean serum Neu5Ac with progressing pregnancy (65). The concentration of serum Neu5Ac ranged from 1.635 ± 0.302 mmol/L at the onset of pregnancy to 2.064 ± 0.494 mmol/L after 37 weeks (65). An inverse pattern is observed in the Neu5Ac content of human milk during lactation. In 1985, Carlson found a mean concentration of oligosaccharide-bound Neu5Ac ranging from 1138 ± 86 mg/L during the first two weeks of lactation to 135 ± 16 mg/L after 10 weeks (1). If one assumes an average milk consumption of 100 kcal/kg body weight/day with an average energy content of 670 kcal/L, this corresponds to a mean daily intake of 170 mg/kg body weight and 20 mg/kg body weight, respectively (1). The concentration of glycoprotein-bound Neu5Ac was also observed to decrease throughout lactation, ranging from 267 ± 24 mg/L in the first two weeks to 73 ± 8 mg/L after 10 weeks (1). Wang et al. found a similar distribution of Neu5Ac in human milk, and noted that milk from mothers of preterm infants contains 13-23% more Neu5Ac at 3 of the 4 lactation stages than milk from mothers delivering full-term infants (2). Based on these observations, it appears that the highest concentration of Neu5Ac is provided before and shortly after birth, during the developmental spurt of brain-mass accretion (5). This may help compensate for the

limited rate of Neu5Ac synthesis in infancy.

It is important to note that the concentration of sialic acid in human milk varies dramatically among individuals when controlled for lactation duration (1, 66). In 2007, Bao et al. observed high variation in sialyloligosaccharides that could not be accounted for by the intrinsic variation in the method employed, suggesting significant biological differences in expression of acidic milk oligosaccharides (66). The composition of oligosaccharides present in human milk is related to the expression and activity of two fucosyltransferases and four phenotypic groups, consistent with the Lewis blood group system, have been identified (67, 68).

Relative to human milk, infant formulas contain very little sialic acid. The sialic acid content of most infant formulas is less than 25% of that found in mature human milk, and typically ranges from 0.05 ± 0.003 mmol/L in soy milk formulas to 0.63 ± 0.12 mmol/L in preterm formulas (2). The sialic acid contained in infant formulas is also more likely to be bound to glycoproteins, rather than free oligosaccharides (2), and the concentration of lipid-bound Neu5Ac has been shown to be two times higher in human milk than cow's milk and formula milk (69). Thus, exclusive consumption of infant formulas may limit Neu5Ac accumulation in the brain during infancy (2, 63, 70).

In 2003, Wang et al. determined the Neu5Ac concentration of 25 samples of postmortem cerebral tissue derived from infants who died of sudden infant death syndrome (70). After adjustment for age at death with sex as a covariate, the average concentration of ganglioside- and glycoprotein-bound Neu5Ac was 32% and 22%

higher, respectively, in breastfed than formula-fed infants (70). Experimental studies employing the use of animals have also demonstrated that an exogenous source of Neu5Ac may play a role in determining the final concentration of Neu5Ac in the brain, and several have noted behavioral and neuronal changes. In 1980, Morgan and Winick demonstrated that Neu5Ac administered through intraperitoneal injection can improve learning performance and increase the concentration of cerebral and cerebellar ganglioside- and glycoprotein-bound Neu5Ac in well-fed and under-nourished rat pups (7, 8). They found similar benefits in rats exposed to environmental stimulation during the first 21 days of postnatal life (8). Early stimulation caused undernourished rats to exhibit a pattern of behavior that more closely resembled that of well-fed rats and resulted in changes in brain composition that persisted into adulthood (8). This suggests that there is more than one mechanistic route to achieve an optimal Neu5Ac concentration in the brain (8, 39).

In 1986, Carlson and House proved that oral administration of Neu5Ac can also increase the concentration of cerebral and cerebellar ganglioside- and glycoprotein-bound Neu5Ac in young rats (71), and, later, Park et al. achieved similar results when feeding rats a ganglioside-enriched diet (72). In 2007, Wang et al. conducted a study in which piglets were randomly allocated to receive a sow milk replacer containing 140, 300, 635, or 830 mg of sialic acid/L from bovine casein glycomacropeptide (CGMP) (5). In a dose-response manner, sialic acid supplementation was associated with faster learning, increased protein-bound sialic acid in the frontal cortex, and amplified expression of two key enzymes involved in

Neu5Ac metabolism, namely PST and GNE (5). Previous studies have revealed that the intracellular concentration of sialic acid regulates the amount of PSA on NCAM (51). The findings of Wang et al. suggest the reverse is also true (5). Increases in the polysialylation of NCAM appear to up-regulate sialic acid synthesis (5). This would explain why sialic acid supplementation correlated with an unexpected increase in GNE expression (5).

Several studies have demonstrated that exogenously administered gangliosides promote neurite outgrowth in vitro and potentiate neurotrophic factor effects (31). Others indicate efficacy of GM1 treatment for individuals with CNS ischemia and spinal cord lesions (31). In 1984, Sabel et al. showed that 30 mg/kg injections of GM1 in adult rats could reduce behavioral deficits after brain injury by enhancing structural reorganization of remaining afferents or preventing secondary neuronal degeneration (73). Similar benefits were observed by She et al. after lead-induced impairment of synaptic plasticity in rats (74). Intraperitoneal administration of gangliosides was shown to significantly increase the amplitude of the population spike and slope of the excitatory post-synaptic potential compared to unsupplemented rats (74). This suggests gangliosides may reverse lead-induced impairments of synaptic plasticity and attenuate the cognitive deficits induced by lead (74).

Genetic and chromosomal mutations affecting Neu5Ac metabolism or the ability to synthesize complex gangliosides result in several neurological abnormalities. Mice lacking complex gangliosides exhibit decreased central myelination, axonal degeneration, loosened myelin sheaths, and demyelination in

peripheral nerves (75, 76). They also display a reduction in nerve conduction velocity and expression of MAG in the CNS (75, 76). In 2004, Simpson et al. found that human autosomal recessive infantile-onset symptomatic epilepsy syndrome is associated with a loss-of-function mutation in GM3 synthase, the key enzyme for the synthesis of all complex gangliosides (77). With the onset of seizure activity, affected children show stagnation in the acquisition of developmental milestones and experience neurological decline (78). In 2007, Gulesserian et al. found a five-fold reduction in the concentration of Neu5Ac synthase in the cortex of fetuses with Down syndrome compared to controls (79). This significantly alters Neu5Ac metabolism and may help account for the cognitive impairment observed in this disorder (79).

Alcohol consumption also appears to affect Neu5Ac metabolism. In 1989, Prasad administered a moderate dose of alcohol to rats throughout gestation and found an increase in the total ganglioside-bound sialic acid, a decrease in ganglioside catabolizing enzymes, and altered proportions of individual ganglioside fractions in the brains of the rat pups at birth (80). Tomás et al. later found that ethanol impairs the uptake of sialic acid in cultured rat astrocytes, but significantly increases the uptake of its precursor, ManNAc (81). This suggests that the carbohydrate transporters located in the plasma membrane are altered by alcohol and may serve as a target for the deleterious effect of ethanol on brain development (81).

Digestion and Absorption of Sialic Acids

Hydrolysis of sialic-acid-containing carbohydrates in the lumen of the small

intestine is achieved by sialidases in the intestinal mucosa (4, 82). Due to their terminal position, the bond between sialic acid residues and the penultimate sugar of the oligosaccharide chain may be cleaved even if the remainder of the chain resists digestion (4). Interestingly, sialidase activity in the middle and distal thirds of the small intestine increases rapidly after birth in several species and is positively correlated with the sialic acid concentration of milk collected from the mothers at corresponding times post partum (82). Autohydrolysis of sialic acid residues in the intestine may also occur because sialic acids are relatively strong organic acids (4).

In 1981, Nöhle and Schauer demonstrated that the intestinal cell walls of rats are highly permeable to free sialic acid (83). N-Acetyl-D-[2-14C,9-3H]neuraminic acid, enzymically prepared from sodium [2-14C]-pyruvate and N-acetyl-D-[6-3H]mannosamine by N-acetylneuraminate lyase in 75% yield, was orally administered to 20 day old fasted mice (83). Approximately 90% of the administered neuraminic acid was absorbed from the intestine in the course of 4 hours, at a rate depending on the retention time of neuraminic acid in the intestine and the mental conditions of the animals (83). This observation may not apply to human digestion and absorption of sialic acid. In 1998, Brand-Miller et al. demonstrated that most human milk oligosaccharides resist digestion in the small intestine and undergo fermentation in the colon (84), and in vitro studies have confirmed that sialic acid is not released from the incubation of human milk oligosaccharides with pancreatic and mucosal enzyme mixtures (85). It is possible that sialidases of bacterial origin cleave sialic acid residues from milk oligosaccharides in the colon, but it is not known if

sialic acid can be absorbed across the colonic mucosa (4).

Casein Glycomacropeptide (CGMP)

CGMP is a C-terminal glycopeptide released from κ-casein by the action of chymosin during cheese making and contains various amounts of covalently linked oligosaccharides, including galactose, N-acetylgalactosamine, and Neu5Ac (86, 87). The oligosaccharide chains of CGMP are attached to the peptide backbone at four threonine residues (numbers 131, 133, 135, and 142) and serine-141 (88). These sites are glycosylated at random and exhibit a large degree of heterogeneity (88). Due to the relative abundance of Neu5Ac, CGMP has been a proposed source of Neu5Ac supplementation for infant formulas. The carbohydrate profile of CGMP varies widely, depending on the isolation method, but CGMP typically provides approximately 60 mg of sialic acid/g (5, 86). Although less abundant, bovine milk oligosaccharides also contain N-glycolylneuraminic acid (89). For this reason, caution should be exercised in the use of CGMP for human consumption.

N-glycolylneuraminic acid

The majority of vertebrates, including the Great Apes, are able to oxidize the α-carbon of the N-acetyl group within CMP-Neu5Ac to produce CMP-Neu5Gc (14, 15, 90). However, due to a frameshift mutation resulting in the lack of a 92 bp region of cDNA, humans are unable to produce the enzyme responsible for catalyzing this reaction, CMP-Neu5Ac hydroxylase (15, 90, 91). Traces of Neu5Gc have been

detected in healthy and malignant human tissues, but no enzymatic activity has been reported (91). It was previously thought that the expression of Neu5Gc was dependent on malignant transformation of the tissue (14). New evidence suggests the small amount of Neu5Gc present in human tissues originates from exogenous sources (90). In a study by Tangvoranuntakul et al., human volunteers consumed 150 mg of porcine submaxillary mucin Sias (95% Neu5Gc) (90). Although a portion was absorbed and eliminated in the urine, a small quantity of free Neu5Gc was metabolically incorporated into newly synthesized glycoproteins (90). This is concerning because humans produce variable amounts of IgA, IgM, and IgG antibodies against Neu5Gc, and Neu5Gc is a common compound found in milk products, lamb, pork, and beef (90). The incorporation of this nonhuman dietary component may potentially create an autoreactive situation (90), and should be addressed if manufacturers attempt to supplement infant formulas with Neu5Ac.

Interestingly, the results obtained by Tangvoranuntakul et al. contrast those obtained in a mouse model, where 98% of radioactively labeled Neu5Gc was absorbed from the intestine and appeared unaltered in the urine 6 hours after oral administration (92). This suggests animal models for Neu5Gc metabolism and excretion may have limited generalizability to humans.

Cholesterol in Infancy

Cholesterol is another component of human milk that is not currently included in infant formulas. Human milk typically contains 10-11 mg of cholesterol per

deciliter, while cows' milk-based and soy-based formulas contain 3-5 mg/dL and 0 mg/dL, respectively (3, 93). Although evidence suggests the cholesterol-synthesizing machinery is well developed in neonates and premature infants (94), it is well documented that infants fed human milk down-regulate cholesterogenesis and have higher plasma cholesterol concentrations than formula-fed infants (3, 95, 96). This suggests an endogenous source of cholesterol may still be important during this period of rapid development.

Cholesterol plays an important role in neuroarchitecture and the transmission of neural impulses. It is a key component of neuronal cell membranes, nerve growth cones, and myelin (9). Cholesterol promotes synaptogenesis and up-regulates genes that influence the development of dendritic spines and neurotransmitter receptors (97). It also serves as a precursor of various metabolically active compounds such as hormones, oxysterols, and bile salts (94). Observational studies in humans have linked total serum cholesterol with measures of cognitive function, such as verbal fluency, concentration, and abstract reasoning, and inborn errors in cholesterogenesis lead to severe congenital defects and mental retardation (12, 94).

In neuronal cell membranes, cholesterol is not uniformly distributed, but is concentrated in lipid rafts. These heterogeneous areas are enriched in gangliosides and hypothesized to function as a platform for signaling pathways (10, 11, 40). The inclusion of cholesterol in lipid rafts increases the fluid character of the plasma membrane and facilitates ganglioside interaction with membrane proteins (26, 39). This suggests the concentration of cholesterol in the membrane may influence the

functional properties of gangliosides, and several studies demonstrate cholesterol depletion leads to perturbation of raft-mediated signaling (40). Interestingly, treatment of astrocytes with the cholesterol-solubilizing detergent methyl-β-cyclodextrin results in the release of extracellular laminin (38). A similar result is observed upon removal of Neu5Ac residues with neuraminidase treatment, suggesting Neu5Ac and cholesterol work in tandem to permit or restrict neurite outgrowth (38).

Chapter 3 Materials and Methods

Overview

The purpose of this study was to determine the impact of glycomacropeptide and dietary cholesterol on cortical ganglioside- and glycoprotein-bound sialic acid accumulation in young rats. Rat pups were allocated to receive one of four dietary glycomacropeptide treatments (0, 14.0, 28.1, and 56.2 g/kg) for two weeks and exposed to 0 or 0.5% dietary cholesterol by weight throughout gestation, lactation, and postweaning. These doses were selected to represent the range of intake typically observed in human milk- and formula-fed infants, and provided approximately 0, 20, 40 and 80 mg sialic acid/kg$\cdot d^{-1}$. The concentration of ganglioside- and glycoprotein-bound sialic acid was determined through established methods (98, 99, 100), and the cholesterol content of the dams' milk was determined according to Rosenthal et al.

Setting

This study was conducted at the University of Kansas Medical Center, Kansas City, Kansas. The animals were raised between November 2006 and June 2007. The analysis of ganglioside- and glyprotein-bound sialic acid and milk cholesterol was performed between August 2007 and November 2008.

Ethics

The study protocol followed the National Research Council's guide for the care and use of laboratory animals and was approved by the Institutional Animal Care

and Use Committee at the University of Kansas Medical Center (2006-1585).

Materials

Female Long-Evans rats (Blue Spruce) were purchased from Harlan Inc. (Indianapolis, IN) (n/group = 12) and bred with proven male breeders (Blue Spruce, Harlan Inc., Indianapolis, IN). Throughout pregnancy and lactation, the rats consumed a nutritionally complete rat diet (AIN-93G, Harlan Teklad, Madison, WI) with or without cholesterol (0.5 or 0% by weight). All litters were culled to 8 pups on postnatal day (P) 1. After weaning, two pups from each litter were housed together and allocated to each of four diets varying in CGMP (0, 14.0, 28.1, and 56.2 g/kg to provide approximately 0, 20, 40 and 80 mg sialic acid/kg$\cdot$d^{-1}, respectively) and the same cholesterol level as their dams. During this portion of the study, the base diet (AIN-93G) was modified slightly with casein and corn starch to ensure similar total protein and carbohydrate concentrations at all CGMP levels **(Table 1)**.

Sialic Acid Supplementation after Early Weaning

In the first experiment, the dams' litters were weaned early on P17. The sialic acid-supplemented diets were fed from weaning to P32, when the pups were sacrificed. The diets were formulated with the Tatua (Morrinsville, New Zealand, 67 mg sialic acid/g) source of CGMP. Experiment one followed the intended experimental design and was conducted from February to June 2007.

Table 1. Composition of the cholesterol-supplemented rat pup diets[1]

	0 mg Neu5Ac/kg/d	20 mg Neu5Ac/kg/d	40 mg Neu5Ac/kg/d	80 mg Neu5Ac/kg/d
AIN-93 G[2]	4650 g	4650 g	4650 g	4650 g
Soybean Oil	350 g	350 g	350 g	350 g
Casein[3]	48.0 g	36.0 g	24.0 g	–
Corn Starch[4]	8.2 g	6.15 g	4.1 g	–
Glycomacropeptide[5]	–	14.05 g	28.1 g	56.2 g
Cholesterol[6]	25 g	25 g	25 g	25 g

[1]The composition of the non-cholesterol diets was identical to the diets shown above, excluding the 25 g of cholesterol at each level.

[2]AIN-93G was purchased from Harlan Tekland (Madison, WI) in powdered form.

[3,4]Glycomacropeptide was 85% protein and 15% carbohydrate by weight. Casein and corn starch were added to compensate for glycomacropeptide at the three lower glycomacropeptide concentrations fed.

[5]Two sources of glycomacropeptide were used for the experiment: Tatua Co-Operative Dairy Company, Morrinsville, New Zealand, 67 mg sialic acid/g and Arla Food Ingredients, Denmark, 55 mg sialic acid/g. The composition shown in Table 1 was for the product from Tatua Co-operative Dairy Co.

[6]Cholesterol was dissolved in the soybean oil prior to mixing with dry ingredients.

Post-weaning Sialic Acid Supplementation

Due to a delay in arrival of the Tatua product (approximately 3 months), we

attempted to salvage the designed study by feeding CGMP later, after the period of maximal ganglioside- and glycoprotein-bound sialic acid accumulation in brain. The diets in the second experiment were formulated with the Arla product (Denmark, 55 mg sialic acid/g), which arrived after all rat pups had exceeded 17 days of age. Litters were weaned on P21 to the diet of their dams **(Table 2)**. Rat pups consumed

Table 2. Composition of dams's non-cholesterol and cholesterol supplemented diets

	Non-Cholesterol Diet	0.5% Cholesterol Diet
AIN-93 G[1]	4650 g	4650 g
Soybean Oil	350 g	350 g
Cholesterol[2]	–	25 g

[1]AIN-93G was purchased from Harlan Tekland (Madison, WI) in powdered form.

[2]Cholesterol was dissolved in the soybean oil prior to mixing with dry ingredients.

the dams' diets until P24 to P33. Pups received their assigned sialic acid-supplemented diets for 14 days and, therefore, were sacrificed between P38 and P47. The decision to analyze the brains in the second experiment was made with the intent of determining how timing of CGMP intake might influence composition.

Quantification of Ganglioside- and Glycoprotein-bound Sialic Acid

On the day of sacrifice, the pups were decapitated and the brains immediately frozen on dry ice. Gangliosides were extracted from the cortex according to the

procedure outlined by Svennerholm and Fredman (98), and the resulting pellet was analyzed for glycoprotein-bound sialic acid (Appendix A). The concentration of ganglioside- and glycoprotein-bound sialic acid was determined using the HCl-resorcinol method (99), as modified by Suzuki (100) (Appendix B). The samples were read using a BioSpec-mini spectrophotometer at 620 nm to negate the contamination of other sugars (98), and the concentrations were calculated by equations derived from standard Neu5Ac solutions (Metreya LLC, Pleasant Gap, PA). A standard curve was analyzed with each set of samples. All sets were balanced to include equal numbers of brains from each of the 8 dietary treatments.

Analysis of Milk Cholesterol

Coagulated milk was removed from the stomach of P1 pups and stored at -80°C in an ultra-low temperature freezer (Legaci®, Asheville, NC) until the day of analysis. After saponification in 10% ethanolic potassium hydroxide (101), the concentration of total milk cholesterol was determined by the method of Rosenthal et al. (102) (Appendix C). Because the treatment of dams was identical through P1 in both experiments and not all litters required culling, the stomach contents from one pup in each available litter from both experiments were analyzed.

Statistical Analysis of Data

Differences in ganglioside-bound sialic acid were determined using a mixed model ANOVA with subject as a random effect (SAS® version 9.2, SAS Institute

Inc., Cary, NC). Even with a daily standard curve, there were effects of day of analysis on sialic acid concentration. Therefore, each day of analysis was treated as a block to decrease the random error introduced by day to day environmental variation (e.g., room temperature and humidity), with cholesterol and concentration of sialic acid intake as main effects. Glycoprotein-bound sialic acid data were analyzed using an ANOVA model that treated cholesterol and day as fixed effects and concentration of sialic acid intake as a fixed covariate interaction (SAS® version 9.2, SAS Institute Inc., Cary, NC). Differences in total milk cholesterol were determined using Student's *t* distribution (Microsoft® Excel 2007).

Chapter 4 Results

Sialic Acid Supplementation after Early Weaning

Seven rat pups in the first experiment died before the date of sacrifice, 3 of which received the diet including cholesterol supplementation (0.5% cholesterol by weight). Glycomacropeptide intake independently and significantly increased (linear trend for dose) cortical ganglioside-bound sialic acid ($p = 0.007$) **(Figure 3)**.

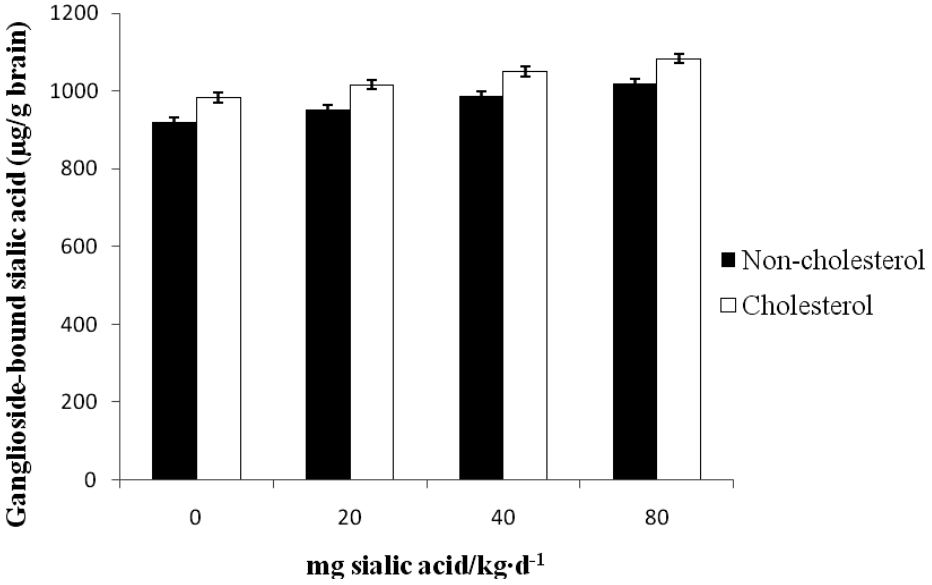

Figure 3. Mean (±SE) concentrations of cortical ganglioside-bound sialic acid in rats fed diets containing 0% and 0.5% cholesterol by weight. Glycomacropeptide independently and significantly increased (linear trend for dose) cortical ganglioside-bound sialic acid ($p = 0.007$) ($n = 10$ at 0 mg sialic acid/kd·d^{-1}; $n = 11$ at all other levels of supplementation). Dietary cholesterol significantly increased ganglioside-bound sialic acid ($p = 0.02$) ($n = 10$ at 40 mg sialic acid/kd·d^{-1}; $n = 11$ at all other levels of supplementation). Specific values are listed in Appendix D.

As Figure 1 shows, exposure to dietary cholesterol during gestation, lactation, and postweaning also significantly increased cortical ganglioside-bound sialic acid ($p = 0.02$). The concentration of cortical glycoprotein-bound sialic acid was not influenced by glycomacropeptide or cholesterol intake. The average concentration of cortical glycoprotein-bound sialic acid was 0.258 and 0.257 mg/g brain (wet weight) in the non-cholesterol and cholesterol supplemented groups, respectively.

In a separate analysis, the influence of dietary cholesterol on cortical cholesterol and protein accumulation was examined. Exposure to dietary cholesterol significantly increased cortical cholesterol and protein in P32 rat pups **(Table 3)**.

Table 3. Mean (±SE) cortical cholesterol and protein per gram of brain in P32 pups

	Cortical Cholesterol	Cortical Protein
Non-Cholesterol Diet	11.71 ± 0.20 mg/g	96.59 ± 1.75 mg/g
Cholesterol Diet	12.61 ± 0.25 mg/g	101.29 ± 1.30 mg/g
P-value[1]	0.006	0.034

[1]Differences in cortical cholesterol and protein were determined using Student's *t* distribution (Microsoft® Excel 2007) (n = 43/group in the analysis of cortical cholesterol; n = 39/group in the analysis or cortical protein).

Post-weaning Sialic Acid Supplementation

In the second experiment, two rat pups receiving the cholesterol-supplemented diet died before the day of sacrifice. Exposure to dietary cholesterol significantly

increased the concentration of glycoprotein-bound sialic acid, but did not affect the concentration of ganglioside-bound sialic acid **(Table 4)**. Glycomacropeptide intake did not influence ganglioside- or glycoprotein-bound sialic acid (data not shown).

Table 4. Mean (± SE) cortical ganglioside- and glycoprotein-bound sialic acid per gram of brain with post-weaning sialic acid supplementation

	Ganglioside-bound Sialic Acid	Glycoprotein-bound Sialic Acid
Non-Cholesterol Diet	1113.39 ± 12.61 μg/g	0.177 ± 0.007 mg/g
Cholesterol Diet	1103.49 ± 12.47 μg/g	0.200 ± 0.008 mg/g
P-value[1]	—	0.030

[1]Differences in ganglioside- and glycoprotein-bound sialic acid were determined using a mixed model ANOVA (SAS® version 9.2, SAS Institute Inc., Cary, NC) (n = 44 in the non-cholesterol diet; n = 42 in the cholesterol-supplemented diet).

The effect of dietary cholesterol on cortical cholesterol and protein accumulation was also examined in the second experiment. Exposure to dietary cholesterol increased cortical protein, but did not significantly influence the concentration of cortical cholesterol **(Table 5)**.

Table 5. Mean (±SE) cortical cholesterol and protein per gram of brain with post-weaning sialic acid supplementation

	Cortical Cholesterol	Cortical Protein
Non-Cholesterol Diet	10.72 ± 0.17 mg/g	124.14 ± 1.38 mg/g
Cholesterol Diet	11.13 ± 0.17 mg/g	129.33 ± 1.37 mg/g
P-value[1]	0.099	0.009

[1]Differences in cortical cholesterol and protein were determined using Student's *t* distribution (Microsoft® Excel 2007) (n = 44 in the non-cholesterol diet; n = 42 in the cholesterol-supplemented diet).

Analysis of Milk Cholesterol

Maternal cholesterol intake significantly increased the cholesterol concentration of milk collected from the stomachs of culled P1 rat pups ($p = 0.0009$). The average concentration of milk cholesterol on P1 was 55.83 ± 9.45 ($\bar{x}$ ± SD; n = 17) and 70.51 ± 13.23 ($\bar{x}$ ± SD; experiment one: n = 16) mg/dL in pups consuming milk from non-cholesterol and cholesterol supplemented dams, respectively.

Chapter 5 Discussion

Sialic Acid Supplementation after Early Weaning

In the first experiment, glycomacropeptide independently and significantly increased cortical ganglioside-bound sialic acid in a dose-response manner, but did not influence glycoprotein-bound sialic acid. The overall concentration of ganglioside-bound sialic acid was similar to that observed in study by Carlson and House, in which rats received Neu5Ac through intraperitoneal injection or intubation (72). This suggests the sialic acid contained in CGMP is bioavailable, and CGMP may be a viable source of sialic acid for supplementation in the future.

In a related study, Park et al. observed similar increases in ganglioside-bound sialic acid when administering a ganglioside-rich diet (0.1% w/w of total fat) to rats for two weeks, beginning on P18 (72). Interestingly, the investigators observed a highly significant reduction in the ratio of cholesterol to ganglioside-bound sialic acid in the brain of rats consuming the ganglioside-rich diet compared with animals fed a control diet (72). A small reduction in the ratio of cholesterol to ganglioside-bound sialic acid was also observed when feeding a diet containing sphingomyelin (1% w/w of total fat) (72), a precursor to the membrane anchor of gangliosides. However, when the investigators examined the overall concentration of cholesterol in the brain, animals fed the sphingomyelin diet did not exhibit any change in total cholesterol content in the brain, while those consuming the ganglioside-rich diet had a significantly lower concentration of brain cholesterol (72). This suggests dietary sialic acid, rather than the lipid portion of gangliosides, plays a functional role in

reducing brain cholesterol. Park et al. suggests dietary gangliosides decrease brain cholesterol by influencing lipid and protein trafficking in lipid rafts (72).

In the present study, dietary cholesterol significantly increased ganglioside-bound sialic acid and cortical cholesterol in P32 rat pups. The concentration of cholesterol in the brain is tightly regulated and not typically influenced by the diet. In attempt to determine whether cholesterol in milk influences growth and the sterol composition of brain, Edmond et al. fed milk substitutes containing variable amounts of cholesterol to artificially reared rat pups from P5 until P15 or 16 (103). The investigators found that the sterol composition of brain was not influenced by the concentration of cholesterol in milk and that cholesterol exogenous to brain, even in a hypercholesterolemic condition, did not gain entry to the brain (103). Based on the findings of Edmond et al. and Park et al., it is possible that dietary cholesterol did not increase the concentration of cortical cholesterol, but sialic acid supplementation reduced cortical cholesterol in rats lacking dietary cholesterol. The results of the present study also indicate that low dietary cholesterol may limit ganglioside-bound sialic acid accumulation in the cortex. Together, these findings suggest cholesterol supplementation may be important during this period of development.

Post-weaning Sialic Acid Supplementation

In the second experiment, glycomacropeptide intake did not influence ganglioside- or glycoprotein-bound sialic acid. The overall concentration of ganglioside-bound sialic acid in both dietary groups was greater than that observed at

the highest level of sialic acid and cholesterol supplementation in the first experiment. This suggests early consumption of sialic acid and cholesterol can accelerate the accumulation of ganglioside-bound sialic acid to a maximal concentration, but that animals will eventually reach this concentration even if they do not consume sialic acid or cholesterol. Although the concentration of cortical ganglioside-bound sialic acid plateaued by 38 to 47 days of age, it is possible that earlier accumulation of sialic acid provides a functional advantage to the developing rat pup.

It is interesting to note that cholesterol supplementation did not significantly increase cortical cholesterol in the second experiment, and the overall concentration of cortical cholesterol was lower in both dietary treatments. If dietary sialic acid truly reduces the concentration of cholesterol in the cortex, supplementation only appears to influence cortical cholesterol during the period of ganglioside-bound sialic acid accumulation. As the concentration of ganglioside-bound sialic acid in both dietary groups plateaued, significant differences in cortical cholesterol were not observed. Although not significant, a slightly lower concentration of ganglioside-bound sialic acid was also related to a higher concentration of cortical cholesterol. The overall reduction in cortical cholesterol observed in the second experiment also suggests a higher level of cholesterol supplementation may be needed during the period of ganglioside-bound sialic acid accumulation to maintain cortical cholesterol.

The overall concentration of glycoprotein-bound sialic acid in both dietary groups was lower than that observed in the first experiment. This is likely due to decreased expression of polysialic acid (PSA) on the neural cell adhesion molecule

(NCAM) (54). After developmental events cease, NCAM shifts from a PSA-rich to a PSA-poor form (21, 54, 56, 57). Thus, one may expect the concentration of glycoprotein-bound sialic acid to decrease as maturation progresses.

Although the overall concentration of glycoprotein-bound sialic acid decreased in the second experiment, it remained significantly higher among rats fed the cholesterol-supplemented diet. While it is possible that dietary cholesterol is slowing the maturational loss of PSA, it seems more probable that cholesterol is exerting an effect on glycoprotein-bound sialic acid by influencing the overall concentration of cortical protein. Exposure to dietary cholesterol increased both cortical protein and glycoprotein-bound sialic acid. However, the proportion of glycoprotein-bound sialic acid to total cortical protein remained stable across both dietary groups. This suggests dietary cholesterol is not directly influencing the concentration of glycoprotein-bound sialic acid in the cortex.

Analysis of Milk Cholesterol

Whatley et al. demonstrated maternal hypercholesterolemia may increase the concentration of milk cholesterol in rabbits (104). However, Green et al. did not observe a significant increase in the milk cholesterol of rats after administering a diet containing 15% lard and 1.5% cholesterol (w/w) from the 18th day of gestation until weaning (105). The results of the present study contrast those obtained by Green et al., demonstrating that cholesterol intake by the dam increases total milk cholesterol. A potential limitation of our analysis is that we were not able to obtain milk directly

from the rats' mammary glands, but removed coagulated milk from the stomachs of P1 rat pups. However, P1 pups are not capable of consuming the maternal diets, and the contents of the pups' stomachs only reflect milk intake and the presence of digestive fluids. Differences in the cholesterol content of digestive fluids among pups receiving milk from cholesterol-supplemented dams seem unlikely.

On the seventh day of lactation, Green et al. observed a milk cholesterol concentration of 71.7 ± 18.2 ($\bar{x}$ ± SD) mg/dL in dams receiving a diet containing 15% lard and 1.5% cholesterol (w/w) from the 18th day of gestation until weaning (105). Although a similar value was obtained for milk collected from the stomachs of culled P1 pups consuming milk from cholesterol-supplemented dams, it is possible that digestive fluids diluted or absorption of water concentrated the milk in the rat pups' stomachs. Therefore, the concentration of cholesterol in the milk collected may not reflect the actual concentration produced by the dams.

Limitations

Other limitations of this study should be noted. The source of CGMP in the modified AIN-93G diets varied between experiments one (Tatua Co-Operative Dairy Company, Morrinsville, New Zealand, 67 mg sialic acid/g) and two (Arla Food Ingredients, Denmark, 55 mg sialic acid/g), and each contained a slightly different proportion of protein-bound sialic acid. Although the concentration of sialic acid was accounted for during the formulation of the four dietary treatments to produce diets with equivalent amounts of sialic acid, the overall composition of the diets varied

slightly. Differences in the bioavailability of sialic acid were also possible. This complicates comparisons made between the results of the two experiments.

The pups in both experiments were fed CGMP as a source of sialic acid after the peak period of ganglioside-bound sialic acid accumulation. They also consumed additional sialic acid and cholesterol that were inherent components of the dams' milk for 17 or 21 days after birth. Both of these factors would be expected to reduce the free capacity for sialic acid and cholesterol intake to influence brain composition.

Finally, we could not determine whether the effects of maternal cholesterol supplementation during gestation influenced brain composition at birth because the P1 rat brains were not stored and available for analysis. Such an analysis may be potentially misleading. The P1 pups were available only from the largest litters, and larger litters were associated with smaller pup size at birth.

Future Research

The pups in experiments one and two were fed CGMP as a source of sialic acid after the peak period of ganglioside-bound sialic acid accumulation. In humans, the total concentration of ganglioside-bound Neu5Ac in the forebrain increases in two distinct phases (59). The first phase occurs between the 13^{th} and 30^{th} week of gestation, and the second occurs from birth to 9 months of age (59). These phases correspond to the time of rapid multiplication of neurons and the extensive establishment of micro-neuronal interconnections, respectively (59). The concentration of ganglioside-bound Neu5Ac in the cerebellum also increases from

birth to 9 months of age (59). Further studies may be needed to determine the effect of maternal and neonatal sialic acid and cholesterol exposure on the accumulation of ganglioside- and glycoprotein-bound sialic acid in the brain during these critical periods. It may also be important to determine if dietary sialic acid influences the expression of fucosyltransferases and the oligosaccharide composition of breast milk.

Conclusions

The results of the present study provide evidence that sialic acid contained in CGMP is bioavailable and can influence brain composition in developing rat pups. They also demonstrate that early consumption of sialic acid and cholesterol can accelerate the accumulation of ganglioside-bound sialic acid to a maximal concentration, but suggest animals will eventually reach this concentration even if they do not consume sialic acid or cholesterol. The findings further suggest dietary cholesterol may be needed during the accumulation of ganglioside-bound sialic acid.

Chapter 6 Summary

This study aimed to determine if CGMP and dietary cholesterol influence cortical ganglioside- and glycoprotein-bound sialic acid accumulation in young rats.

Rat pups were allocated to one of four glycomacropeptide intakes, providing approximately 0, 20, 40 and 80 mg sialic acid/kg•d^{-1} for two weeks, and exposed to 0 or 0.5% dietary cholesterol by weight throughout gestation, lactation, and postweaning. The concentration of ganglioside- and glycoprotein-bound sialic acid was determined through established methods (98, 99, 100), and the cholesterol content of the dams' milk was determined according to Rosenthal et al.

Milk cholesterol concentration was increased by maternal cholesterol intake from 55.8 to 70.5 mg/mL ($p = 0.0009$). In pups sacrificed on P32, CGMP increased cortical ganglioside-bound sialic acid concentration in a dose-response manner (mg/g; p for trend = 0.007). In the same experiment, dietary cholesterol independently increased ganglioside-bound sialic acid ($p = 0.02$). In pups sacrificed between P38 and P47, cholesterol intake increased glycoprotein-bound sialic acid ($p = 0.030$).

The results of the present study provide evidence that the sialic acid contained in glycomacropeptide is bioavailable. They demonstrate that early consumption of sialic acid and cholesterol can accelerate the accumulation of ganglioside-bound sialic acid to a maximal concentration, and they suggest dietary cholesterol may be important during the period of ganglioside-bound sialic acid accumulation.

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APPENDIX A

Procedure for the Extraction of Gangliosides from Cortical Tissue

Procedure for the Extraction of Gangliosides from Cortical Tissue

1. Homogenize one hemisphere of cortex in an equal volume (w/v) of lysis buffer with a Sonics Vibra Cell for 1 minute at 20% maximum speed.

2. Remove 250 µL of sample prepared as per number 1. Add 875 µL of distilled water and vortex to mix. Add 4 mL of 1:2 chloroform/methanol.

3. Cap tube tightly with a Teflon-lined lid and vortex mixture for 30 minutes at room temperature.

4. Centrifuge at 3300 rpm (1380 x G) for 30 minutes.

5. Pour supernatant into clean 16 x 125 mm screw-top culture tube with a marking at 11 mL. Cap tightly with a Teflon-lined lid.

6. Reextract brain residue with an additional 5 mL of 4:8:3 chloroform/methanol/water (v/v). Cap tube tightly and vortex mixture for 30 minutes at room temperature. Centrifuge at 3300 rpm (1380 x G) for 30 minutes.

7. Combine supernatant with first extraction and add chloroform/methanol/water 4:8:3 to 11 mL mark.

8. Add 1.7 mL of water to the remaining 10 mL of extracted brain lipids to achieve a final chloroform/methanol/water ratio of 4:8:5.6.

9. Mix by inversion 30 – 40 times. Let mixture sit overnight at 4°C or centrifuge 15 minutes at 3300 rpm (1380 x G) to separate phases.

10. Remove upper phase (contains gangliosides) and transfer to a new 16 x 125 mm screw-top culture tube.

11. Reextract the lower phase by adding 1.25 mL of methanol and 0.81 mL of 0.01 M KCl. Mix well by shaking.

12. Centrifuge for 15 minutes at 3300 rpm (1380 x G). Combine with first upper phase and dry under a stream of nitrogen in a 37°C water bath.

13. Dissolve residue of upper phase in 250 µL of chloroform/methanol/water 60:30:4.5 (v/v). Allow solution to sit at room temperature for 24 hours.

14. Centrifuge to spin down any precipitate, and remove two 40 uL aliquots for sialic acid analysis. Store remaining sample at -20°C.

APPENDIX B

Procedures for the Quantification of Ganglioside- & Glycoprotein-Bound Sialic Acid

Procedure for the Quantification of Ganglioside-Bound Neu5Ac

1. Remove two 40 µL aliquots of each extracted ganglioside sample and place in two separate 16 x 125 mm screw-top culture tubes with Teflon-lined lids.

2. Dry under a stream of nitrogen in a 37°C water bath.

3. While samples are drying, prepare standards (0, 2, 5, 10, 20, 30, and 40 µg/0.5 mL) using the volume of 0.20 µg/µL stock sialic acid solution and water specified for each standard in the following table:

Standard (µg/500 µL)	Volume of 0.20 µg/µL Stock Sialic Acid Solution	Volume of Water
0	0 µL	500 µL
2	10 µL	490 µL
5	25 µL	475 µL
10	50 µL	450 µL
20	100 µL	400 µL
30	150 µL	350 µL
40	200 µL	300 µL

4. After samples are dry, dissolve residue in 0.5 mL of distilled water. Vortex for 5 minutes to mix.

5. Add 0.5 mL of the resorcinol-HCl reagent to each sample and standard.

   Preparation of resorcinol-HCl reagent:

   Combine 4.875 mL of distilled water with 125 µL of 0.1 M copper (II) sulfate pentahydrate and 5 mL of 2% resorcinol (2 g of resorcinol dissolved in 100 mL of distilled water). (Stable several months at 4°C)

   Add 40 mL of concentrated HCl (37%, 12 N) and mix well.

   Resorcinol-HCl reagent must be prepared at least 4 hours before use. (Stable 1 week at 4°C.)

6. Vortex samples for 5 minutes.

7. Heat culture tubes for 25 minutes in a boiling water bath to develop color.

8. After heating, cool tubes in running water; add 1.5 mL of butylacetate/butanol (85/15, v/v). Vortex for 5 minutes, then place in ice water for 15 minutes.

9. Centrifuge for 1 minute at 3300 rpm (1380 x G).

10. Place culture tubes in ice water again until photometric readings are taken.

11. To obtain absorbance value, pipet the upper, colored phase into a cuvette using a transfer pipet and read at 620 nm with a BioSpec-mini spectrophotometer.

Procedure for the Quantification of Glycoprotein-Bound Neu5Ac

1. Dry each glycoprotein sample under a stream of nitrogen in a 37°C water bath to remove any residual extraction solvent.

2. While samples are drying, prepare standards (0, 2, 5, 10, 20, 30, and 40 µg/0.5 mL) using the volume of 0.20 µg/µL stock sialic acid solution and water specified for each standard in the following table:

Standard (µg/500 µL)	Volume of 0.20 µg/µL Stock Sialic Acid Solution	Volume of Water
0	0 µL	500 µL
2	10 µL	490 µL
5	25 µL	475 µL
10	50 µL	450 µL
20	100 µL	400 µL
30	150 µL	350 µL
40	200 µL	300 µL

3. After samples are dry, dissolve residue in 1.0 mL of distilled water. Vortex for 5 minutes to mix.

4. Add 0.5 mL and 1.0 mL of the resorcinol-HCl reagent to each standard and sample, respectively.

   Preparation of resorcinol-HCl reagent:

   Combine 4.875 mL of distilled water with 125 µL of 0.1 M copper (II) sulfate pentahydrate and 5 mL of 2% resorcinol (2 g of resorcinol dissolved in 100 mL of distilled water). (Stable several months at 4°C)

   Add 40 mL of concentrated HCl (37%, 12N) and mix well.

   Resorcinol-HCl reagent must be prepared at least 4 hours before use. (Stable for 1 week at 4°C.)

5. Vortex samples for 5 minutes.

6. Heat culture tubes for 25 minutes in a boiling water bath to develop color.

7. After heating, cool tubes in running water; add 1.5 mL and 3.0 mL of butylacetate/butanol (85/15, v/v) to each standard and sample, respectively. Vortex for 5 minutes, then place in ice water for 15 minutes.

8. Centrifuge for 1 minute at 3300 rpm (1380 x G).

9. Place culture tubes in ice water again until photometric readings are taken.

10. To obtain absorbance value, pipet the upper, colored phase into a cuvette using a transfer pipet and read at 620 nm with a BioSpec-mini spectrophotometer.

APPENDIX C

Procedure for the Analysis of Total Cholesterol in Milk

Procedure for the Analysis of Total Cholesterol in Milk

1. Thaw frozen milk samples and sonicate for 1 minute at 20% maximum amplitude (Sonics Vibra Cell).

2. Pipet 25 µL of the homogenized milk samples into separate 16 x 125 mm screw-top culture tubes.

3. Add 250 µL of ethanolic potassium hydroxide solution to each tube.

   Preparation of potassium hydroxide solution:

   > Dissolve 5 g of reagent grade KOH in 10 mL of water to form 33% KOH (w/v) solution.

   Preparation of ethanolic potassium hydroxide solution:

   > Add 6.06 mL of 33% KOH (w/v in H_20) to 13.94 mL of absolute ethanol to form a 10% KOH solution (w/v).

   Solution must be prepared immediately before use!

4. Cap each tube tightly with a Teflon-lined lid and vortex 3 minutes to mix.

5. Incubate tubes in a water bath set at 37 – 40ºC for 55 minutes.

6. After cooling to room temperature, add 0.5 mL of petroleum ether and vortex 5 minutes to mix.

7. Add 250 µL of water to each tube and vortex vigorously for 1 minute.

8. Centrifuge tubes at 3300 rpm (1380 x G) for 3 minutes or until the emulsion breaks and two clear layers form.

9. Freeze the lower phase in an acetone-dry ice bath for 3 minutes and pipet upper phase (petroleum ether layer) into new, separate 16 x 125 mm screw-top culture tube. (The extraction can be interrupted at this stage, and the tubes capped and stored at 4ºC until analysis.)

10. Place the tubes in a water bath set at 60ºC and evaporate the petroleum ether layer under a stream of nitrogen.

11. While samples are drying, prepare standards (0, 5, 10, 15, 20, 25, and 30 µg/2.5 mL) using the volume of 0.5 µg/µL stock cholesterol solution and glacial acetic acid specified for each standard in the following table:

Standard (µg/2.5 mL)	Volume of 0.5 µg/µL Stock Cholesterol Solution	Volume of Glacial Acetic Acid
0	0 µL	2.50 mL
5	10 µL	2.49 mL
10	20 µL	2.48 mL
15	30 µL	2.47 mL
20	40 µL	2.46 mL
25	50 µL	2.45 mL
30	60 µL	2.44 mL

12. Reconstitute the residue with 2.5 mL of glacial acetic acid and mix well to dissolve. (If needed, warm the acetic acid to 70ºC to aid dissolution.)

13. Add 2 mL of the color reagent to the samples and standards; mix gently, but thoroughly. (Avoid vigorous shaking, which produces bubbles.)

    Note: If the acetic acid was warmed in the previous step, allow the samples to cool to room temperature before adding the color reagent.

    Preparation of the iron stock solution:

    Dissolve 2.5 g of $FeCl_3 \cdot 6H_20$ in 100 mL of phosphoric acid (85%). (Stable indefinitely at room temperature.)

    Preparation of the color reagent:

    Dilute 16.0 mL of iron stock solution to 200 mL with concentrated sulfuric acid (98%). (Stable 8 weeks at room temperature if protected from atmospheric moisture.)

14. Determine the absorbance 30 minutes after mixing and read at 560 nm.

APPENDIX D

Mean Concentrations of Cortical Ganglioside-bound Sialic Acid in Rats Fed Diets Containing 0% and 0.5% Cholesterol by Weight

Mean Concentrations of Cortical Ganglioside-bound Sialic Acid in Rats Fed Diets Containing 0% and 0.5% Cholesterol by Weight

mg sialic acid/kg/d	Non-Cholesterol Diet	Cholesterol Diet
0	919.76 μg/g	983.80 μg/g
20	953.08 μg/g	1017.13 μg/g
40	986.41 μg/g	1050.46 μg/g
80	1019.73 μg/g	1083.78 μg/g

Differences in ganglioside-bound sialic acid were determined using a mixed model ANOVA (SAS® version 9.2, SAS Institute Inc., Cary, NC). Glycomacropeptide independently and significantly increased (linear trend for dose) cortical ganglioside-bound sialic acid ($p = 0.007$) ($n = 10$ at 0 mg sialic acid/kd·d^{-1}; $n = 11$ at all other levels of supplementation). Dietary cholesterol significantly increased ganglioside-bound sialic acid ($p = 0.02$) ($n = 10$ at 40 mg sialic acid/kd·d^{-1}; $n = 11$ at all other levels of supplementation).